Inhibition of the Proteasome Activity by Gallium(III) Complexes Contributes to Their Anti–Prostate Tumor Effects

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Abstract

The investigation of metal-based complexes with potential antitumor activity has been of paramount importance in recent years due to the successful use of cisplatin against various cancers. Gallium(III) and subsequently developed gallium(III)-containing complexes have shown promising antineoplastic effects when tested in a host of malignancies, specifically in lymphomas and bladder cancer. However, the molecular mechanism responsible for their anticancer effect is yet to be fully understood. We report here for the first time that the proteasome is a molecular target for gallium complexes in a variety of prostate cancer cell lines and in human prostate cancer xenografts. We tested five gallium complexes (1–5) in which the gallium ion is bound to an N’O asymmetrical ligand containing pyridine and substituted phenolate moieties in a 1:2 (M/L) ratio. We found that complex 5 showed superior proteasome inhibitory activity against both 26S proteasome (IC50, 17 μmol/L) and purified 20S (IC50, 16 μmol/L) proteasome. Consistently, this effect was associated with apoptosis induction in prostate cancer cells. Additionally, complex 5 was able to exert the same effect in vivo by inhibiting growth of PC-3 xenografts in mice (66%), which was associated with proteasome inhibition and apoptosis induction. Our results strongly suggest that gallium complexes, acting as potent proteasome inhibitors, have a great potential to be developed into novel anticancer drugs.

Introduction

Since the approval of cisplatin nearly 30 years ago, renewed efforts have focused on developing metal-based chemotherapeutic agents with improved clinical efficacy and reduced overall toxicity (1). Gallium is a naturally occurring metal that has been investigated in clinical trials against a number of malignancies including lymphomas and bladder cancer (2–6). It has also shown efficacy against a diverse set of disorders such as accelerated bone resorption and autoimmune and infectious diseases (2). Gallium is currently approved by Food and Drug Administration as an anticancer drug for their anticancer effect has not been fully understood. Here, we show for the first time that synthetic gallium complexes, especially 5, 4, and 3, are able to target and inhibit proteasomal activity and induce apoptosis in various prostate cancer cell lines. The ability to inhibit proteasome activity was indicated by the accumulation of ubiquitinated proteins and the proteasomal target protein p27. The proteasome inhibitory potency of complex 5 was shown both in in vitro studies and in a human prostate tumor

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Gallium complexes as Tumor Proteasome Inhibitors

Figure 1. Gallium complexes inhibit the chymotrypsin-like activity of purified 20S proteasome and cellular 26S proteasome. A, chemical structures of gallium complexes. B, inhibition of the chymotrypsin (CT)-like activity of purified 20S proteasome. C, inhibition of proteasome activities in intact human prostate C4-2B cells. C4-2B cells were treated with 50 μmol/L of the five gallium complexes for 18 h, followed by measurement of proteasomal chymotrypsin-like activities.

Materials and Methods

Materials. The gallium complexes 1 to 5 were synthesized as previously described (28). Hoechst 33258 and cremophor were purchased from Sigma-Aldrich. Purified rabbit 20S proteasome and fluorogenic peptide substrates Suc-LLVY-AMC and Ac-DEVD-AMC were obtained from Calbiochem, Inc. Peptide substrate Z-GGL-AMC was from BIOMOL International LP. Apoptag Peroxidase In Situ Apoptosis Detection Kit was from Chemicon International, Inc.

Cell cultures and whole-cell extract preparation. Human prostate cancer cells, LNCaP, C4-2B, and PC-3, were grown in RPMI 1640 supplemented with 10% fetal bovine serum and maintained at 37°C and 5% CO2. A whole-cell extract was prepared as previously described (25, 29).

Inhibition of purified 20S proteasome activity. Purified rabbit 20S proteasome (17.5 ng) was incubated in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 7.5) with 10 μmol/L of fluorogenic substrate Suc-LLVY-AMC for 2 h at 37°C. After incubation, production of hydrolyzed AMC group was measured by fluorescence at 520 nm.

Inhibition of the proteasome activity in intact cells. C4-2B cells were cultured in a 96-well plate (1 × 104 per well) and treated with various concentrations of gallium complexes for 18 h. After an additional 2-h incubation with the fluorogenic peptide substrate Z-GGL-AMC, which is specific for the proteasomal chymotrypsin-like activity, production of hydrolyzed AMC groups was measured as described above.

Caspase-3 and proteasomal chymotrypsin-like activity assays. Proteins extracted from cells or tumor tissue were incubated for 1 h at 37°C in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 7.5) with 10 μmol/L of fluorogenic substrate Suc-LLVY-AMC (for chymotrypsin-like activity in cultured cells), Z-GGL-AMC (for chymotrypsin-like activity in tumor tissues), or Ac-DEVD-AMC (for caspase-3/caspase-7 activity) as previously described (31).

Cellular and nuclear morphology analysis. A Zeiss Axiovert 25 microscope was used for all microscopic imagings, either with phase contrast for cellular morphology or with fluorescence for nuclear morphology with Hoechst 33258 staining as previously described (31).

Human prostate tumor xenograft experiments. Five-week-old male athymic nude mice were purchased from Taconic Research Animal Services and housed under pathogen-free conditions according to Wayne State University animal care guidelines. The protocols of animal experiments were reviewed and approved by Institutional Laboratory Animal Care and Use Committee of Wayne State University. PC-3 cells (2 × 105) were injected s.c. at one flank of the mice. The mice were then randomly grouped and injected s.c. daily with solvent (PBS/cremophor/ethanol/DMSO = 5:2.7:1.3:1) as a control (n = 9), 20 mg/kg of complex 5 (n = 9), or 20 mg/kg of ligand 5 (L5; n = 5) for 29 days. Tumor size was measured every other day using calipers. Tumor volume (V) was determined by the equation V = (L × W2)/2, where L is the length and W is the width of the tumor.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, immunostaining, and H&E assays. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using

xenograft model. In contrast, complexes 1 and 2 or gallium salt alone was unable to sustain these effects under the same in vitro conditions. Our results show a novel approach to use gallium complexes as proteasome inhibitors and apoptosis inducers in prostate cancer therapy.
in situ apoptosis detection kit and immunostaining of p27 were done as previously described (29). H&E staining in tumor tissues was done following manuactory protocols (29). Briefly, paraffin-embedded sample slides were deparaffinized and hydrated and then stained with hematoxylin for 1 min. After rinsing, the slides were stained with eosin for 1 min, rinsed, and coverslips were mounted onto slides with Permount.

**Statistical analysis.** Statistical analysis was done with Microsoft Excel software. Student’s t test for independent analysis was applied to evaluate differences between treatments and control.

**Results**

Structural relationships of several synthetic gallium complexes with their activities to inhibit purified 20S proteasome and cellular 26S proteasome. We have previously reported that certain copper complexes are potent proteasome inhibitors (29, 30, 32). We hypothesized that gallium complexes might be similarly capable of targeting and inhibiting the proteasome in human tumor cells. To test this hypothesis, we analyzed proteasome inhibitory potencies of five gallium complexes (Fig. 1A) under cell-free conditions and found that complexes 3, 4, and 5 inhibited chymotrypsin-like activity of the purified 20S proteasome with IC₅₀ values of 46, 27, and 16 μmol/L, respectively (Fig. 1B). In contrast, complex 2 showed very weak inhibitory potential, whereas complex 1 had no effect at the highest concentration used (50 μmol/L; Fig. 1B). The rank of the inhibitory potencies of the gallium complexes against the purified 20S proteasome is 5 > 4 > 3 > 2 > 1, consistent with the order of proteasome inhibitory potency of the complexes.

To determine the potencies of these complexes to inhibit chymotrypsin-like activity in intact cells, androgen-independent human prostate cancer C4-2B cells (33) were plated into a 96-well plate and treated with each of the five complexes for 18 h, followed by measurement of proteasome activity. We found that the IC₅₀ values of complexes 3, 4, and 5 in intact C4-2B cells were 48, 28, and 17 μmol/L, respectively (Fig. 1C), consistent with their inhibitory potencies against the purified 20S proteasome. Complexes 1 and 2 again showed only slight inhibitory effect (Fig. 1C).

**Inhibition of proteasomal chymotrypsin-like activity by gallium complexes in androgen-independent human prostate cancer cells is associated with down-regulation of androgen receptor and induction of apoptosis.** To confirm the ability of these gallium complexes to inhibit the proteasomal activity in prostate cancer cells, C4-2B cells were treated with each complex at 50 μmol/L concentrations for 18 h. The cells were harvested and used for cell extract preparation, followed by measurement of the proteasomal chymotrypsin-like activity and accumulation of ubiquitinated proteins. Complexes 1 to 5 were found to inhibit 7.5%, 14%, 38%, 62%, and 81% of the proteasomal activity, respectively (Fig. 2A, left). Additionally, high levels of accumulated ubiquitinated proteins were detected in C4-2B cells treated with complexes 3, 4, and 5 (Fig. 2B, left).

It has been shown that proteasome inhibition could down-regulate androgen receptor expression (34). Therefore, if gallium complexes were able to inhibit proteasomal activity, we would expect a decrease in androgen receptor expression level. Indeed, the results in Fig. 2B showed that gallium complexes 2 and 3, but not complex 1, down-regulated androgen receptor protein (3 > 2 > 1), whereas complexes 4 and 5 completely abrogated androgen receptor protein expression (Fig. 2B, left). These results remained consistent with the order of proteasome inhibitory potency of the gallium complexes.

It has also been shown that inhibition of the proteasomal chymotrypsin-like activity in malignant cells could result in the induction of apoptosis (24, 25). To investigate whether the proteasomal inhibition and androgen receptor level reduction by gallium complexes are associated with apoptotic cell death, PARP cleavage and morphologic and nuclear changes were measured in the same experiment. The results showed that treatment with gallium complexes 3, 4, and 5 caused apoptosis-specific PARP cleavage (Fig. 2B, left), whereas complexes 1 and 2 failed to do so (Fig. 2B). Consistently, morphologic changes (shrunked cells and characteristic apoptotic blobbing Fig. 2C, top) and the presence of apoptotic nuclei after Hoechst staining (condensed or punctuated and brighter nuclei; Fig. 2C, bottom) were observed in the cells treated for 18 h with complexes 4 and 5. Whereas complex 3 induced only moderate level of apoptotic cellular and nuclear changes (Fig. 2C), complexes 1 and 2 showed some to no activity (Fig. 2C). These results show that the induction of apoptosis in C4-2B cells by gallium complexes is associated with the inhibition of the proteasomal chymotrypsin-like activity, and that the apoptosis-inducing potency of these compounds was also ranked as 5 > 4 > 3 > 2 > 1.

To investigate the effects of different concentrations of gallium complexes on prostate cancer cells, we selected the most potent complex 5 and treated C4-2B cells at various concentrations (10, 25, and 50 μmol/L), followed by measurement of proteasome inhibition, androgen receptor protein level, and apoptosis induction. The results showed that complex 5 inhibited the proteasome in a dose-dependent manner as measured by proteasomal chymotrypsin-like activity (Fig. 3A) and accumulation of ubiquitinated proteins and proteasome target protein p27 (ref. 35; Fig. 3B). Decrease in androgen receptor level and apoptosis-specific PARP cleavage were also induced in a dose-dependent manner (Fig. 3B).

**Inhibition of proteasomal chymotrypsin-like activity by gallium complexes in androgen-dependent human LNCaP prostate cancer cells is associated with down-regulation of androgen receptor and induction of apoptosis.** After we showed the ability of the gallium complexes to inhibit proteasome activity and induce apoptosis in androgen-independent C4-2B prostate cancer cells, we tested the effect of the same gallium complexes on the androgen-dependent LNCaP cell line (33). LNCaP cells were treated with 50 μmol/L, concentration of each complex for 18 h, followed by measurement of the proteasome activity, androgen receptor protein level, and apoptosis induction. We found that gallium complexes 2, 3, 4, and 5 inhibited chymotrypsin-like activity of the proteasome by 12%, 39%, 62%, and 79%, respectively (Fig. 2A, right), whereas complex 1 showed only a slight effect compared with solvent control (Fig. 2A, right). Consistent with the inhibition of the proteasomal chymotrypsin-like activity, significantly increased levels of ubiquitinated proteins were detected in LNCaP cells treated with complexes 3 to 5 (Fig. 2B, right). Complex 5 was also found to be most potent in decreasing androgen receptor level, whereas complex 1 had almost no effect (Fig. 2B, right). In the same experiment, treatment with gallium complexes 3, 4, and 5 resulted in massive cell detachment (data not shown) and PARP cleavage (Fig. 2B, right). Additionally, complexes 3 to 5 induced caspase-3/caspase-7 in a time-dependent manner (data not shown), whereas complexes 1 and 2 showed only a slight effect. These results show that in androgen-dependent LNCaP cells, these gallium complexes have the same rank of proteasome inhibitory, androgen receptor-suppressing, and apoptosis-inducing potencies as in androgen-independent C4-2B cells.

**Time-dependent proteasome inhibition and apoptosis induction by gallium complex 5 in androgen-independent human prostate cancer PC-3 cells.** Our results revealed that complex 5,
of all tested complexes, had superior proteasome inhibitory and apoptosis-inducing abilities in androgen receptor–dependent prostate cancer C4-2B and LNCaP cells (Figs. 1–3). To study the effect of these compounds in androgen receptor–independent prostate cancer cells, PC-3 cells were treated with 50 μmol/L of complexes 1 to 5 for 18 h, followed by measurement of chymotrypsin-like activity (A); Western blot analysis of ubiquitinated proteins (Ub-Prs), androgen receptor (AR), and PARP (B); and staining for cellular morphologic and nuclear changes in C4-2B (C). DMSO (DM) was used as solvent control.

Proteasome inhibitory and apoptosis-inducing activities of gallium complex 5 and L5 in human prostate cancer xenografts. Our in vitro data show that gallium complexes 3, 4, and 5 act as proteasome inhibitors and apoptosis inducers in cultured human prostate cancer cells and that complex 5 is the most potent (Figs. 1–4). To investigate whether compound 5 could also inhibit the proteasome and induce apoptosis in vivo, we used mice bearing human prostate tumor xenografts. PC-3 cells were implanted s.c. into male nude mice and allowed to grow until the appearance of a palpable tumor (~120 mm³). The mice were then randomly grouped and injected s.c. daily with solvent, 20 mg/kg complex 5, or 20 mg/kg L5 for 29 days. At the end of the trial, the mice were sacrificed and tumor tissues were harvested and used for multiple assays. Measurement of tumor size showed that tumor growth was inhibited by 66% in complex 5–treated mice and by only 30% in L5-treated mice, compared with the control mice (Fig. 5A). Therefore, complex 5 possesses potent antitumor property in vivo. However, antitumor activity of L5 was also observed (see below).

To determine if the observed antitumor effects of complex 5 and L5 are associated with proteasome inhibitory and apoptosis-inducing activities in vivo, the prepared tissue samples were used for several assays. Figure 5B shows inhibition of the proteasomal chymotrypsin-like activity by 65% in complex 5–treated tumors, compared with the control, whereas L5-treated tumors showed...
only 31% inhibition. Consistently, accumulation of ubiquitinated proteins and p27 was found in tumors treated with complex 5, as measured by Western blot analysis (Fig. 5C). L5-treated tumors were also able to accumulate ubiquitinated proteins and p27, but to a lesser extent (Fig. 5C). This suggests the possibility of L5 combining with endogenous metal species, such as copper, which forms partial proteasome-inhibiting complexes (see Discussion). Increased accumulation of p27 protein in tumors treated by complex 5 or L5 was further confirmed by immunohistochemistry assay, which showed the increase of p27-positive cells by 64%, 25%, and 7% in tumors treated with complex 5, L5, or solvent, respectively (Fig. 6A).

Furthermore, we found that the inhibition of the proteasomal chymotrypsin-like activity in tumors treated with complex 5 or L5 was associated with apoptosis, as shown by induction of caspase-3/caspase-7 activity (Fig. 5D) and the appearance of cleaved PARP fragment (Fig. 5C). Induction of apoptosis in tumors treated with complex 5 or L5 was further confirmed by the presence of TUNEL-positive cells (78% and 19% in complex 5- and L5-treated tumors, respectively; Fig. 6B) and high levels of condensed apoptotic nuclei detected by H&E staining (76% and 23% in complex 5- and L5-treated tumors, respectively; Fig. 6C). Whereas L5 alone was able to induce some level of apoptosis, complex 5 was much more potent, showing superior tumor growth inhibition. We monitored the body weight of mice from each group and the average readings were 25.9, 25.7, and 25.5 g from the mice treated with the solvent, L5, and complex 5, respectively. The data of the body weight showed that there was no toxicity of L5 and complex 5 observed in the treated mice. Taken together, these results clearly show that complex 5 was able to target the proteasome in vivo, resulting in induction of apoptotic cell death.

Discussion

The main problems with conventional metal-based chemo-therapeutic strategies are nonspecific interactions and the acquisition of drug resistance. The screening and subsequent development of copper-based compounds as antitumor agents have shown promising preclinical results, thereby highlighting a
potential novel therapeutic strategy (29, 36). Gallium complexes have been investigated in clinical trials and ongoing studies are trying to optimize drug disposition and pharmacokinetic parameters (1, 10, 17).

The established antitumor activity and therapeutic potential of gallium complexes have renewed our interest in exploring their mechanisms of action (1, 5, 10). Although many studies are investigating biological effects of gallium, they are mainly focused on a transferrin-mediated mode of action, with subsequent inhibition of DNA synthesis (1, 5, 11). Some studies with gallium have been implicated in the programmed cell death pathway concomitant with iron deficiency and sustained gallium exposure (3, 15). However, the mechanism of action triggered by gallium complexes remained mainly unclear.

Because gallium complexes showed inhibition of cell proliferation against cisplatin-resistant neuroblastoma cells (28), we decided to further investigate their biological activities against prostate cancer cells and tumors. We show here that some of these gallium complexes are very potent apoptosis inducers in androgen-dependent and androgen-independent prostate cancer cells. Moreover, we reveal the 26S proteasome as their target, which represents an important step in delineating their mechanism of action.

The gallium complexes investigated here were synthesized by using asymmetrical ligands containing pyridine and 2,6-substituted phenol moieties (Fig. 1A). Whereas their cell killing activities have been well established, their coordination mode and structure-activity relationship are not well understood. In the current study, we have found that complex 5 is much more potent than complexes 1 to 4, suggesting that L5 possesses certain characteristics that, after coordination with gallium, provide an optimal biological response. This optimal response may be governed by the strong π electron-donating iodine group. Considering that all ligands used to synthesize complexes 3 to 5 contain electron-withdrawing halogen substituents, only their π-donating ability could relate to their antitumor effects (I > Br > Cl). Iodine retains very weak electron-withdrawing ability but is a very strong π-donating group, which can activate the ring system of the gallium complex and influence its ability to bind the proteasome. However, the influence of the coordination mode of the metal ion and phenol substitute group on its therapeutic effect is purely speculative at this point. We also showed that L5 is able to bind other metals, such as copper, and

![Figure 5. Complex 5 inhibits tumor growth in mice bearing PC-3 xenografts.](image)
that L5 mixed with copper potently inhibited the proteasome and induced apoptosis in human prostate cancer cells (data not shown). It has been reported that tumor tissue contains an elevated level of copper (37, 38). Therefore, one possible explanation for some tumor growth inhibition observed in the mice treated with L5 could be an effect of complex made between L5 and copper.

We also found that the gallium(III) chloride was relatively nontoxic and that concomitant treatment with complex 5 and iron(III) chloride partially precluded the cytotoxic effect of complex 5 (data not shown). Therefore, we propose that gallium complexes, rather than gallium ions sequestered from the complex, are taken up through the transferrin receptor–mediated pathway. However, the exact mechanism for uptake of gallium complexes and their intracellular trafficking and binding to the proteasome need to be further investigated.

The most important aspects in our study were to investigate whether these gallium complexes were active in vivo and to verify their molecular target(s). Therefore, we tested the effects of complex 5, the most potent gallium complex, and its ligand, L5, in mice bearing human PC-3 xenografts. Our data showed that treatment with complex 5 caused a significant inhibition of PC-3 tumor growth in nude mice (Fig. 5A). Importantly, the antitumor activity of complex 5 was associated with the proteasomal activity inhibition (Fig. 5B), accumulation of the proteasome target proteins p27 (Figs. 5C and 6A), and induction of apoptosis, shown by caspase-3/caspase-7 activation, PARP cleavage, TUNEL positivity, and nuclei condensation (Figs. 5 and 6). Taken together, our current study strongly suggests that gallium complexes, by acting as potent proteasome inhibitors, have great potential to be developed as novel anticancer drugs.
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